Patent Application
Docket No. MA-20CCCD4
Serial No. 10/633,023

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Art Unit | : | 1656 |
| Applicants | : | Schnepf, Schwab, Payne, Narva, and Foncerrada |
| Filed | : | July 31, 2003 |
| For | : | NEMATICIDAL PROTEINS |

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

37 CFR §1.132 DECLARATION OF DR. KENNETH E. NARVA

Sir:

DR. KENNETH E. NARVA hereby declares:

THAT, I received the following degrees:

1980-1985: Ph.D. (8/85), Microbiology, Louisiana State University, Department of Microbiology, Baton Rouge, LA; studied the microbiology and molecular biology of lignin degradation in the lab of Dr. V.R. Srinivasan.

1976-1980: B.S. (5/80), Biology, Central Michigan Univ., Mt. Pleasant, MI

THAT, I have been employed professionally as follows:

Group Leader, Dow AgroSciences, 2005-present. Responsible for leading an18-member Cell Biology Department focused on plant transformation capabilities, transgenic crop production and plant cell-based protein production. Additional personal responsibilities include cross-functional leadership of the program to discover novel insect control proteins.

Technical Leader, Dow AgroSciences, 1999-2003. Responsible for supervising a nine-member team of molecular biologists focused on discovery and development of transgenic traits for agronomically important crops. The primary functions of the team are gene identification, isolation, sequencing, and expression in microbial and plant vectors. The team is recognized for having delivered five input traits currently in commercial development for

control of important insect pests corn and cotton. Additional personal responsibilities include membership on product development teams, assisting Legal in preparation of patent applications, and participating in product registration processes.

Research Scientist, Mycogen Corporation, 1989-1999. Molecular biology of *Bacillus thuringiensis* toxins and their development as transgenic and foliar biopesticides. Responsible for supervising a gene cloning and expression team of five people focused on the discovery and development of *Bacillus thuringiensis* toxins for use as biopesticides. The primary functions of the team are the molecular characterization (mapping and PCR), cloning, DNA sequencing, heterologous microbial expression and transgenic design of *B.t.* toxin genes for both Mycogen Biopesticides and Plant Sciences divisions.

THAT, I have the following research experience:

Postdoctoral Research Associate, American Cyanamid Company, 1987-1989. Molecular biology of antibiotic biosynthesis in Streptomycetes. Studied the transcriptional control of undecylprodigiosin (Red) biosynthesis in *Streptomyces coelicolor* A3(2) as a model system in the lab of Dr. Jerald S. Feitelson.

Postdoctoral Research Associate, University of Nebraska-Lincoln, Department of Plant Pathology, 1985-1987. Studied the molecular biology of DNA restriction and modification systems encoded by *Chlorella* algal viruses in the lab of Dr. James L. Van Etten.

THAT, I have been the author or co-author on numerous scientific publications;

THAT, I am an inventor on numerous patents;

THAT I am an inventor of the subject application, and that I am aware of issues raised in the Office Action dated April 4, 2006;

AND, being thus duly qualified as an expert in the field of the invention who is familiar with the prosecution of the subject patent application, do hereby declare:

The subject 52A1 gene (SEQ ID NO:7) was obtained by DNA hybridization procedures as described in Example 5 of the specification. As described in that Example, recombinant phage containing the 52A1 gene (in a *Sau*3A insert) were plated onto *E. coli* cells, plaques were screened by hybridization to DNA probes, and hybridizing phage were plaque-purified and used to infect liquid cultures of *E. coli* cells for "...isolation of phage DNA by standard procedures (Maniatis et al.)."

Maniatis *et al.* is also cited in Example 7 for "isolation of phage DNA by standard procedures", and the full citation of Maniatis *et al.* (1982) is provided in that Example.

The actual procedures used for these hybridization conditions were as described on pages 326-328 (attached) of Maniatis (1982) in the section entitled "Hybridization to Nitrocellulose Filters Containing Replicas of Bacteriophage Plaques or Bacterial Colonies," particularly in Step 7 on page 327.

Furthermore, with the subject gene (SEQ ID NO:7) and toxin in hand, one skilled in the art could readily use related hybridization probes to screen collections of *Bacillus thuringiensis*, for example, for related genes and toxins.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or of any patent issuing thereon.

Further, declarant sayeth not.

Kenneth E. Narva

Kenneth E. Narva

8-31-06

Date

Attachment: Pages 326-328 of Maniatis (1982)

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## HYBRIDIZATION TO NITROCELLULOSE FILTERS CONTAINING REPLICAS OF BACTERIOPHAGE PLAQUES OR BACTERIAL COLONIES

The following protocol is designed for (a) two 20-cm × 30-cm nitrocellulose filters or (b) 30 circular, 82-mm-diameter filters. Appropriate adjustments should be made to the volumes when carrying out hybridization reactions with different numbers or sizes of filters.

1. Float the baked filters on the surface of a tray of 6× SSC until they have become thoroughly wetted from beneath. Submerge the filters for 5 minutes.

2. Transfer the filters (a) to a rectangular, flat-bottomed plastic box (22 cm × 32 cm) or (b) to a circular, glass crystallizing dish. Stack the filters on top of one another.

3. Add (a) 300 ml or (b) 100 ml of prewashing solution. Incubate at 42°C for 1–2 hours.

   In this and all subsequent steps, the circular filters in the crystallizing dish should be agitated on a rotating platform so that they do not stick to one another. The large, rectangular filters may be stationary.

   The prewashing solution removes from the filters any absorbed medium, fragments of agarose, or loose bacterial debris.

   *Prewashing solution*

   50 mM Tris · Cl (pH 8.0)
   1 M NaCl
   1 mM EDTA
   0.1% SDS

4. Pour off the prewashing solution. Incubate the filters for 4–6 hours at 42°C in (a) 100–150 ml or (b) 60 ml of prehybridization solution.

   The filters should be completely covered by the prehybridization solution. During prehybridization, sites on the nitrocellulose filter that bind single- or double-stranded DNA nonspecifically become saturated by unlabeled, salmon sperm DNA, SDS, or components in the Denhardt's solution. When using $^{32}$P-labeled cDNA or RNA as a probe, poly(A) should be included in the prehybridization solution and hybridization solutions at a concentration of 1 μg/ml to prevent the probe from binding to T-rich sequences that are found fairly commonly in eukaryotic DNA.

   *Prehybridization solution*

   50% formamide
   5× Denhardt's solution
   5× SSPE
   0.1% SDS
   100 μg/ml denatured, salmon sperm DNA

After all the components have dissolved, centrifuge the prehybridization solution at 1000*g* at 15°C for 15 minutes or filter it through Whatman 1MM paper using a Buchner funnel. Sterilize the solution by filtration through disposable Nalgene filters. Store frozen at −20°C in 25-ml aliquots.

*Formamide.* Many batches of reagent-grade formamide are sufficiently pure to be used without further treatment. However, if any yellow color is present, the formamide should be deionized by stirring on a magnetic stirrer with Dowex XG8 mixed-bed resin for 1 hour and filtering twice through Whatman 1MM paper. Deionized formamide should be stored in small aliquots under nitrogen at −70°C.

*Denhardt's solution (50×)*

| | |
|---|---|
| Ficoll | 5 g |
| polyvinylpyrrolidone | 5 g |
| BSA (Pentax Fraction V) | 5 g |
| $H_2O$ | to 500 ml |

*20× SSPE.* See page 314.

*Denatured, salmon sperm DNA.* This is prepared as follows: Dissolve the DNA (Sigma Type-III sodium salt) in water at a concentration of 10 mg/ml. If necessary, stir the solution on a magnetic stirrer for 2-4 hours at room temperature to help the DNA to dissolve. Shear the DNA by passing it several times through an 18-gauge hypodermic needle. Boil the DNA for 10 minutes and store at −20°C in small aliquots. Just before use, heat the DNA for 5 minutes in a boiling-water bath. Chill it quickly in ice water.

5. Denature the $^{32}$P-labeled probe DNA by heating for 5 minutes to 100°C. Add the denatured probe to the prehybridization solution covering the filters. Incubate at 42°C until 1-3× $C_0t_{1/2}$ is achieved (see page 325). During the hybridization, the containers holding the filters should be tightly closed to prevent loss of fluid by evaporation.

6. After the hybridization is completed, discard the hybridization solution. Wash the filters 3-4 times, for 5–10 minutes each wash, in a large volume (300–500 ml) of 2× SSC and 0.1% SDS at room temperature. Invert the filters at least once during washing. At no stage during the washing procedure should the filters be allowed to dry.

7. Wash the filters twice for 1–1.5 hours in (a) 500 ml or (b) 300 ml of a solution of 1× SSC and 0.1% SDS at 68°C. At this point, the background is usually low enough to put the filters on film. If the background is still high or if the experiment demands washing at higher stringencies, immerse the filters for 60 minutes in (a) 500 ml or (b) 300 ml of a solution of 0.2× SSC and 0.1% SDS at 68°C.

8. Dry the filters in air on a sheet of Whatman 3MM paper at room temperature. Tape the filters (numbered side up) onto sheets of 3MM paper and place pieces of tape marked with radioactive ink at several locations on the 3MM paper. These markers serve to align the autoradiograph with the filters.

   Radioactive ink is made by mixing a small amount of $^{32}P$ with a waterproof black ink. We find it convenient to make the ink in three grades: very hot (> 2000 cps on a minimonitor); hot (> 500 cps on a minimonitor); and cool (> 50 cps on a minimonitor). Use a fiber-tipped pen to apply ink of the desired hotness to the pieces of sticky tape.

9. Cover the Whatman 3MM paper and filters in Saran Wrap. Apply to X-ray film (Kodak XR or equivalent) and expose overnight at −70°C with an intensifying screen (see pages 470ff).

10. After development, align the film with the filters using the marks left by the radioactive ink. Use a fiber-tipped pen to mark the film with the position of the asymmetrically located dots on the numbered filters. Tape a piece of tracing paper to the film. Mark the position of positive hybridization signals onto the tracing paper. Also mark (in a different color) the positions of the asymmetrically located dots. Remove the tracing paper from the film. Identify the positive colony or plaque by aligning the dots on the tracing paper with those on the agar plate.

    Some batches of nitrocellulose filters swell and distort during hybridization so that it becomes difficult to align the two sets of dots. This problem can be alleviated to some extent by autoclaving the filters before use (see pages 304–305).

11. Each positive plaque should be picked as described in Chapter 2 and placed into 1 ml of SM containing a droplet of chloroform. Often, the alignment of the filters with the plate does not permit identification of a single hybridizing plaque. In this case, an agar plug containing several plaques is picked. An aliquot (usually 50 $\mu$l of a $10^{-2}$ dilution) of the bacteriophages that elute from the agarose plug should be replated so as to obtain approximately 500 plaques on an 85-mm plate. These plaques should then be screened a second time by hybridization. A single, well-isolated, positive plaque should be picked and used to make a plate stock (see page 64).

    Each positive bacterial colony should be picked with a sterile toothpick into 2 ml of medium containing the appropriate antibiotics. The bacteria are then replated so as to obtain approximately 300 colonies on an 85-mm plate. If the original colony was picked from an uncrowded area of the original plate, a small number of the secondary colonies should be picked and grown overnight in 2-ml cultures. The plasmids in these bacterial cultures should be isolated and analyzed by one of the methods described in Chapter 11. If the original colony was picked from a very crowded area of the original plate, it may be worthwhile screening the secondary colonies by hybridization before isolating and analyzing plasmid DNA.